Biochemical Pharmacology, Vol. 20, pp. 897–907. Pergamon Press, 1971. Printed in Great Britain

REACTION OF MONO-BROMO DERIVATIVES OF CYCLOPENTANE, CYCLOHEXANE AND CYCLOHEPTANE AND OF RELATED COMPOUNDS WITH GLUTATHIONE *IN VIVO* AND THE NATURE OF THE SULPHUR-CONTAINING METABOLITES EXCRETED

S. P. JAMES, D. J. JEFFERY,* R. H. WARING and D. A. WHITE†
Department of Biochemistry, University of Birmingham

(*Received* 21 *August* 1970; *accepted* 30 *October* 1970)

Abstract—Rats and rabbits have been dosed with cyclopentyl, cyclohexyl and cycloheptyl bromide and also the corresponding cycloalkenes and cycloalkene epoxides. The excretion of glucosiduronic acids and ethereal sulphates by rabbits dosed with the above compounds has been measured, the extent of glucosiduronic acid formation from cycloalkyl bromides and cycloalkenes being found to increase with increasing ring size. The above cycloalkyl bromides, cycloalkenes and cycloalkene epoxides were fed to rats, and the level of total liver glutathione measured 1, 2 and 4 hr after dosing, cycloalkenes of a series being found to give the greatest reduction in liver glutathione, and cycloalkyl bromides the least. The excretion of mercapturic acids has been measured in both rabbit and rat, the 3-hydroxymercapturic acids being the major metabolites from the cycloalkyl bromides and cycloalkenes, while the epoxides only form the 2-hydroxy isomers. These mercapturic acids have been identified by mass spectrometry and comparison with synthetic compounds. Evidence is presented to show that these hydroxylations are probably carried out by a system involving cytochrome P-450.

GLUTATHIONE transferases have been described which catalyze the reaction between several types of compounds and glutathione. They include glutathione-S-alkyl transferase[1] for which iodomethane was the first substrate described, glutathione-S-epoxide transferase[2] for which a range of epoxides are substrates while a further transferase catalyses the reaction between glutathione and some unsaturated compounds.[3] It is generally accepted that the glutathione conjugates formed *in vivo* are converted to S-substituted cysteines; these may undergo a variety of reactions one of which is acetylation to form mercapturic acids. With some compounds hydroxymercapturic acids[4–6] are also formed but the stage in the metabolic pathway at which hydroxylation occurs has not been established in all cases.

In these experiments rats were dosed with monobromo derivatives of cyclopentane, cyclohexane and cycloheptane and also the corresponding cycloalkenes and epoxides; the effect of these compounds on the level of glutathione in rat liver is reported. The sulphur-containing metabolites excreted by rabbits and rats dosed with the same compounds have been examined and the suitability investigated of the monobromocycloalkanes and cycloalkenes as substrates for the microsomal hydroxylating system. Preliminary reports of some of this work have been given.[7,8]

* Present address: Beecham Research Laboratories, Medicinal Research Centre, The Pinnacles, Fourth Avenue, Harlow, Essex.
† Present address: Chemistry Dept., University of Calgary, Calgary 44, Alberta, Canada.

Table 1. Properties of synthetic mercapturic acids

	M.p	Found (%)				Required (%)			
		C	H	N	S	C	H	N	S
N-Acetyl-S-cyclopentyl-L-cysteine	159–161	52·1	7·4	5·8	13·9	51·95	7·4	6·1	13·85
**N*-Acetyl-S-(*cis*-2-hydroxycyclopentyl)-L-cysteine	170–171	62·1	9·4	6·4	7·4	61·7	9·35	6·5	7·5
**N*-Acetyl-S-(*trans*-2-hydroxycyclopentyl)-L-cysteine	180–181	62·0	9·6	6·4	7·55	61·7	9·35	6·5	7·5
N-Acetyl-S-cyclohexyl-L-cysteine	145–146	54·1	7·65	5·6	13·1	53·9	7·8	5·7	13·3
**N*-Acetyl-S-(*cis*-2-hydroxycyclohexyl)-L-cysteine	152–153	62·2	9·2	6·25	7·1	62·4	9·5	6·3	7·3
**N*-Acetyl-S-(*trans*-2-hydroxycyclohexyl)-L-cysteine	174–175	62·4	9·6	6·3	7·4	62·4	9·5	6·3	7·3
N-Acetyl-S-cycloheptyl-L-cysteine	146–148	55·6	8·2	5·7	12·6	55·6	8·1	5·4	12·35
**N*-Acetyl-S-(*cis*-2-hydroxycycloheptyl)-L-cysteine	200–201	63·1	9·8	6·0	7·4	62·9	10·0	6·1	7·0
**N*-Acetyl-S-(*trans*-2-hydroxycycloheptyl)-L-cysteine	211–212	63·0	9·6	6·2	7·3	62·9	10·0	6·1	7·0

* Isolated as dicyclohexylammonium salts.

MATERIALS

All melting points are uncorrected. Elemental analyses were carried out by Weiler & Strauss, Oxford, or by the Analytical Chemistry Department, University of Birmingham. Bromocyclopentane, bromocyclohexane, bromocycloheptane, cyclopentene, cyclohexene and cycloheptene were purchased as were a mixture of 1,3 cyclohexane diols and 1,4 cyclohexane diol. The cyclic alkene epoxides used were prepared by the method of Goodman *et al.*[9] and had physical properties in agreement with those recorded in the literature. Cycloalkyl mercapturic acids were prepared by the interaction of the bromocycloalkanes and *N*-acetylcysteine according to the method described by James and White.[6] *Trans*-(2-hydroxycycloalkyl)-mercapturic acids were similarly prepared from the cycloalkene epoxides and *N*-acetylcysteine while the isomeric *cis* 2-hydroxymercapturic acids were obtained by reacting cyclo-

TABLE 2. R_f VALUES AND RETENTION TIMES OF REFERENCE COMPOUNDS

	R_f values in solvents				Retention times (min) in columns	
					5% QF-1	10% SE-30
	A	B	C	D*	205°	205°
N-Acetyl-S-cyclopentyl-L-cysteine	0·58	0·81	0·45	1·0	5·4	4·1
N-Acetyl-S-(*cis*-2-hydroxycyclopentyl)-L-cysteine	0·42	0·70	0·32	0·35	10·1	6·8
N-Acetyl-S-(*trans*-2-hydroxycyclopentyl)-L-cysteine	0·51	0·76	0·37	0·41	11·2	8·0
N-Acetyl-S-(*cis*- & *trans*-3-hydroxycyclopentyl)-L-cysteine	0·42	0·69	0·32	0·29	16·1 19·0	9·7 14·4
					203°	203°
N-Acetyl-S-cyclohexyl-L-cysteine	0·73	0·86	—	1·0	7·1	6·0
N-Acetyl-S-(*cis*-2-hydroxycyclohexyl)-L-cysteine	0·57	0·81	—	0·46	15·7	10·1
N-Acetyl-S-(*trans*-2-hydroxycyclohexyl)-L-cysteine	0·55	0·80	—	0·37	19·0	11·7
N-Acetyl-S-(*cis*- & *trans*-3-hydroxycyclohexyl)-L-cysteine	0·51	0·69	—	0·24	20·5	15·6
	0·44	0·77	—	0·39	23·9	16·2
N-Acetyl-S-(4-hydroxycyclohexyl)-L-cysteine	0·51	0·70	—	0·38	28·4	17·0
					210°	210°
N-Acetyl-S-cycloheptyl-L-cysteine	0·62	0·84	0·39	1·0	7·0	5·8
N-Acetyl-S-(*cis*-2-hydroxycycloheptyl)-L-cysteine	0·58	0·80	0·32	0·56	14·4	10·6
N-Acetyl-S-(*trans*-2-hydroxycycloheptyl)-L-cysteine	0·57	0·81	0·32	0·59	16·6	12·4
N-Acetyl-S-(*cis*- and *trans*-3-hydroxycycloheptyl)-L-cysteine	0·46	0·55	0·30	0·43	23·8 25·2	14·2 17·8

* Rate of movement relative to appropriate mercapturic acid.

The solvents for chromatography were A, butan-1-ol–ethanol–aq. NH_3(sp. gr. 0·88)–water (10:10:1:4, by vol.); B, butan-1-ol–water–acetic acid–butylacetate (24:10:5:2, by vol.); C, butan-1-ol saturated with aq. 2N NH_3; D, butan-2-one saturated with aq. 2N NH_3.

alkyl 1,2-bromohydrins, prepared by the method of Goodman *et al.*[9] with *N*-acetyl cysteine. 2-Hydroxymercapturic acids were isolated as dicyclohexyl-ammonium salts. The mercapturic acids and the dicyclohexyl-ammonium salts of the hydroxymercapturic acids were recrystallized to constant melting point from a mixture of ethanol and acetone except for cyclohexylmercapturic acid for which a mixture of acetone and petroleum ether (b.p. 40–60°) was used. The melting points and elemental analyses are recorded in Table 1 and the R_f values and retention times are recorded in Table 2. The mass spectra of the methyl esters of the *cis*- and *trans*-2-hydroxycyclopentyl, -cyclohexyl and -cycloheptyl mercapturic acids were determined. Peaks at *m/e* corresponding to the molecular ion were either very small or not present but in all the compounds peaks were seen corresponding to loss of H_2O from the molecular ion and large peaks corresponding to the loss of $C_2H_3O_2$.

Other monohydroxy derivatives of all three mercapturic acids were prepared but not isolated. Their chromatographic properties are recorded in Table 2. 1-Bromocyclopent-2-ene prepared according to the method of Hatch and Bachmann[10] was hydroxylated using the procedure of Brown and Geoghegan[11] to give a yellow oil containing 1-bromocyclopentan-2- and 1-bromocyclopentan-3-ols. This was not purified but was reacted with *N*-acetylcysteine as previously described. The product contained, in addition to the 2-hydroxymercapturic acid previously described, a substance, the chromatographic properties of which are given in Table 2, and which is probably the 3-hydroxy derivative. 3-Hydroxycycloheptylmercapturic acid was similarly prepared but not isolated. 3-Hydroxy and 4-hydroxy derivatives of cyclohexylmercapturic acid were prepared from a mixture of *cis*- and *trans*-1,3-diol and from 1,4-cyclohexane diol. These were converted to the bromohydrin by interaction with acetylbromide in acetic anhydride at 120° according to the method of McAsland and Horsewill.[12] Methanol was added to the reaction product and the filtered solution evaporated to an oil which was reacted with *N*-acetylcysteine. The hydroxymercapturic acids formed were separated from excess *N*-acetylcysteine by streaking on to 3MM chromatography paper. The chromatograms were developed in solvent A (see Table 2) and the appropriate zones eluted with aqueous methanol.

[^{35}S]-*labelled yeast.* Yeast was grown on a medium containing sodium [^{35}S] sulphate as described by Williams and Dawson.[13]

METHODS

Chromatography. Details of the solvent mixtures used for paper chromatography on 3MM paper are given in Table 2. The chromatograms were developed by the descending method with solvents A and B for 16 hr, with solvent C for 24 hr and with solvent D for up to 48 hr. The R_f values for the reference compounds in solvents A, B and C are given in Table 2 and in solvent D the rates of movement of the hydroxycycloalkylmercapturic acids relative to that of the appropriate cycloalkylmercapturic acid are recorded. The detecting reagents used were the $K_2Cr_2O_7$–$AgNO_3$ reagent[14] for bivalent sulphur compounds and the chloroplatinate reagent[15,16] for divalent sulphur compounds and sulphoxides. Gas–liquid chromatography was carried out on a Pye series 104, dual column flame ionization chromatograph (W. G. Pye & Co., Cambridge). The columns used consisted of acid-washed silanized Chromosorb W coated with either 5% QF-1 (column E) or 10% S.E.30 (column F) as the stationary

phase. The mercapturic acids and hydroxymercapturic acids were applied to the column as their methyl esters prepared as previously described.[17]

Determination of total glutathione. The method of Martin and McIlwain[18] was used for the determination of total glutathione, the oxidized GSSG present being reduced by pre-incubation with the glutathione reductase of yeast. The experiments to determine the change in the glutathione content of the liver following administration of the compounds studied to the rat were carried out as described by Barnes, James and Wood.[19] The recovery of glutathione added to liver extracts was 103 per cent $\pm$ 9.

Determination of metabolites excreted in urine

Sulphate. This was determined by the method of Folin.[20]

Glucosiduronic acid. The modified naphtharesorcinol method described by Bray *et al.*[21] was used except that the colours were read on an EEL colorimeter with filter 601.

Mercapturic acids. These were determined directly on samples of urine by the paper chromatographic method previously described[22] using solvents A or D. Calibration curves were constructed using the dicyclohexylammonium salts of the appropriate 2-hydroxycycloalkyl mercapturic acids. In the cyclopentyl and cycloheptyl series of compounds the gas chromatographic method[18] of determination was also used with cyclohexylmercapturic acid as an internal standard.

Experiments with rats fed with [^{35}S]*-labelled yeast.* These were carried out as described by James and White.[6]

Experiments with microsomal hydroxylating system. Rats dosed with phenobarbitone for 3 days as described by Remmer[23] were killed after starving for 24 hr and the microsomal fraction prepared[24] from the liver. The difference spectra produced on addition of the bromocycloalkanes and cycloalkenes to the microsomal fraction were observed using an SP700 spectrophotometer.

Experiments with rat liver slices. The bromocycloalkanes were incubated with rat liver slices.[25] A parallel set of digests was incubated after saturating with carbon monoxide. After incubation the digests were homogenized, acidified and filtered. The filtrates were continuously extracted with ether and the extract examined by GLC after esterification with diazomethane.

RESULTS

Glutathione content of livers

The total glutathione content of the livers of control and dosed rats is given in Table 3. The values for control rats agreed with those previously reported.[19] The recovery of glutathione added to liver extracts was the same for control and dosed animals. It can be seen that at a dose level of 3 m-moles/kg the cycloalkenes produced a greater fall in the level of total glutathione in the liver than did the corresponding epoxides when these values are compared 2 hr after the dose. The effect of the bromocycloalkanes was less marked at the same dose level. The administration of cyclohexene resulted in a rapid drop in the total glutathione level suggesting that the compound reaches the liver cells very quickly.

TABLE 3. EFFECT OF SOME MERCAPTURIC ACID PRECURSORS ON THE LEVEL OF TOTAL GLUTATHIONE IN RAT LIVER

Compound administered	Dose (m-moles/kg)	Total glutathione (mg/100 g liver) after:—			
		0·5 hr	1·0 hr	2·0 hr	4·0 hr
Water only*		184 (176,191)[2]	187 187[1]	186 (186–187)[4]	186 (175–209)[3]
Bromocyclopentane	1·8	—	216 (201,230)[2]	202 (199,205)[2]	174 (169,179)[2]
	3·0	—	—	126 (106–157)[3]	—
Bromocyclohexane	1·6	—	172 (155,189)[2]	194 (175,209)[4]	132 (131,134)[2]
	3·0	—	—	176 (173–178)[2]	
Bromocycloheptane	2·2	—	178 (171,185)[2]	145 (115,175)[2]	82 (64,99)[2]
	3·0	—	—	135 (138,132)[2]	—
Cyclopentene	1·9†	—	173 (162–193)[3]	139 (102,175)[2]	125 (120,129)[2]
	3·0†	—	—	131 (131)[2]	—
Cyclohexene	3·0	67 (65,70)[2]	23 (17–27)[4]	39 (15–67)[9]	25 (22,28)[2]
Cycloheptene	2·6	—	117 (105–125)[4]	98 (91,106)[2]	—
	3·0	—	—	84 (83,85)[2]	—
Cyclopentene epoxide	3·8	—	194 (193,195)[2]	166 (150,173)[2]	200 (180,219)[2]
Cyclohexene epoxide	3·0	—	56 (30–115)[4]	82 (48,115)[2]	97 (82,111)[2]
Cycloheptene epoxide	1·5‡	—	—	96 (95,97)[2]	148 (146,149)[2]

* These results are in agreement with those reported[19] previously when the average values for total glutathione were 189 at 0 hr; 173 at 0·5 hr; 177 at 2 hr and 175 at 4 hr.

† Absorbed dose.

‡ Low dose used because of toxicity of compound.

Doses, suspended in water, administered at 0 hr to rats which had been fasted for 19 hr. Results are expressed as means with ranges in parentheses; the superior figure indicates the number of experiments. Control animals were given water by stomach tube.

Reaction with cytochrome P_{450}

The difference spectra observed when the bromocycloalkanes and cycloalkenes were added to the microsomal suspension showed a trough at 420 nm and a peak at about 385 nm indicating that all the compounds were Type I substrates.

Characterization of metabolites excreted in urine

Derivatives of cyclopentane. (a) *Bromocyclopentane.* The results obtained with rabbits and rats were qualitatively the same. The $K_2Cr_2O_7$–$AgNO_3$ and chloroplatinate reagents revealed three sulphur-containing spots on paper chromatograms developed

in solvents A, B and C. Two of these corresponded to cyclopentylmercapturic acid and *trans*- (2-hydroxycyclopentyl) mercapturic acid. The third spot, which represented the major sulphur-containing metabolite, corresponded to the synthetic compound believed to be 3-hydroxycyclopentyl mercapturic acid. Samples of the ethereal extracts were methylated with diazomethane and examined by GLC using columns E and F; peaks having the same retention time as cyclopentyl and *trans*-2-(hydroxycyclopentyl) mercapturic acid were detected. A sample of the methyl ester of the metabolite corresponding to cyclopentylmercapturic acid was separated using the preparative GLC column and examined by mass spectrometry. A peak at *m/e* 245 shown by mass measurement to correspond to the formula $C_{11}H_{19}O_3NS$, was prominent. This corresponds to the molecular ion of the methyl ester of cyclopentylmercapturic acid. The major sulphur-containing metabolite was separated from the ethereal extracts by streaking on to Whatman 3MM paper which was developed in solvent A. The zone containing this metabolite was cut out and eluted, and the eluate passed down a column of Zeo-Karb 225 (H^+ form) then evaporated, methylated and examined by GLC. Methyl esters of *cis*-(2-hydroxycyclopentyl) mercapturic acid and 3-hydroxycyclopentylmercapturic acid were detected, the former in small amount.

(*b*) *Cyclopentene.* *Cis*- and *trans*-(2-hydroxycyclopentyl) mercapturic acid were detected and 3-hydroxycyclopentylmercapturic acid was found to be the main sulphur-containing metabolite in the ethereal extracts of the urine of dosed rabbits and rats. The main metabolite was methylated, separated by GLC and examined by mass spectrometry. The top mass peak was seen at *m/e* 243 corresponding to loss of water from the parent molecular ion.

(*c*) *Cyclopentene epoxide.* Two sulphur-containing metabolites were detected corresponding to *cis*- and *trans*-(2-hydroxycyclopentyl) mercapturic acid.

Derivatives of cyclohexane. (*a*) *Bromocyclohexane.* Cyclohexylmercapturic acid in trace amounts was detected as a metabolite of bromocyclohexane in the rabbit. The main sulphur-containing metabolite was identified with the synthetic hydroxycyclohexylmercapturic acid presumed to be the 3-hydroxy isomer. Smaller amounts of *cis*-2-hydroxycyclohexyl mercapturic acid and the 4-hydroxy isomer were shown to be present by GLC. In the rat only 3-hydroxycyclohexylmercapturic acid was detected.

(*b*) *Cyclohexene.* With both rabbit and rat traces of cyclohexylmercapturic acid and 2-hydroxycyclohexylmercapturic acid were detected but the main metabolite was identified with that formed from bromocyclohexane, i.e. 3-hydroxycyclohexylmercapturic acid; both isomers were detected. This metabolite was separated by paper chromatography in solvent A. The free acid was prepared using Zeo-Karb 225 and methylated. The mass spectrum of the methyl ester showed small peaks at *m/e* 275 and 257 corresponding to the molecular ion and a species produced by loss of H_2O from the molecular ion respectively. A prominent peak occurred at *m/e* 216 corresponding to loss of C_2H_3O from the molecular ion.

(*c*) *Cyclohexene epoxide.* Paper chromatography revealed the presence of *cis*-(2-hydroxycyclohexyl)mercapturic acid in the ethereal extracts of the urine of dosed rabbits and rats and traces of *trans*-(2-hydroxycyclohexyl)mercapturic acid were present in extracts of rabbit urine. These results were confirmed by GLC. It was found that the synthetic methyl ester of *trans*-2-hydroxycyclohexyl mercapturic acid was unstable.

Cycloheptane derivatives. (*a*) *Bromocycloheptane.* Paper chromatography of the

Table 4. Excretion of mercapturic acid and hydroxymercapturic acids by dosed rabbits and rats

Compound administered	Dose m-mole/kg	Rabbit						Rat			
		Glucuronide (%)	Ethereal sulphate (%)	Mercapturic acid (%)				Mercapturic acid (%)			
				Alkyl	*cis*-2-OH Alkyl	*trans*-2-OH Alkyl	3-OH-Alkyl	Alkyl	*cis*-2-OH Alkyl	*trans*-2-OH Alkyl	3-OH-Alkyl
Bromocyclopentane	1·8	27* (16–38)[6]	13* (12–16)[4]	1·8 (1·6–2·0)[4]	0·8 (0·6–0·9)[4]	0·8 (0·6–0·9)[4]	21·3 (19·6–22·2)[4]	1·0 (0·8–1·2)[4]	1·8 (1·4–2·2)[4]	1·7 (1·3–2·1)[4]	17·4 (16·4–18·0)[4]
Bromocyclohexane	1·6	61* (35–84)[3]	9* (8–10)[3]	tr	tr	ND	10·3§ (9·9–11)[3]	ND	ND	ND	11·1 (10·2–12·9)[3]
Bromocycloheptane	1·5	78* (69–85)[6]	19* (17–22)[3]	1·2 (1·2–1·3)[3]	ND	ND	5·5 (5·5)[3]	0·6 (0–1·1)[3]	ND	ND	4·2 (3·9–4·8)[3]
Cyclopentene	3·0†	0 (0)[5]	2·3 (2·1–2·5)[3]	0₄	0·4 (0·3–0·4)[4]	0·4 (0·3–0·5)[4]	11·9 (10·9–13·0)[4]	ND	0·6 (0·3–1·0)[4]	0·6 (0·4–0·9)[4]	9·7 (8·5–11·0)[4]
Cyclohexene	2·0	20 (17–25)[3]	3·5 (3·1, 3·8)[2]	tr	tr	ND	25·4§ (20·6–28·0)[3]	tr	tr	ND	12·7 (12·3–13·0)[3]
Cycloheptene	2·9	60 (63–66)[3]	9·2 (8·3–10·1)[3]	tr	ND	ND	6·4 (6·2–6·5)[3]	tr	tr	ND	6·22 (6·1–6·4)[3]
Cyclopentene epoxide	2·4	15 (10–18)[4]	1·5 (0–4·5)[3]	0₄	0·8 (0·8–0·9)[3]	0·8 (0·7–0·8)[3]	ND	ND	1·2 (1·1–1·3)[3]	1·3 (1·1–1·4)[3]	ND
Cyclohexene epoxide	3·0	37 (30–40)[3]	11·3 (10·9, 11·6)[2]	0₃	2·8 (2·2–3·2)[3]	tr	ND	ND	7·6 (6·3–8·7)[3]	tr	ND
Cycloheptene epoxide	1·5†	16 (14–18)[3]	14·4 (12·8–16·3)[3]	0₃	0·6 (0·6–0·7)[3]	0·7 (0·6–0·7)[3]	ND	ND	1·0 (0·9–1·1)[3]	0·9 (0·8–1·0)[3]	ND

* From previous results.
† In two experiments it was shown that 37 and 48 per cent of dose was exhaled in 3 hr.
‡ Toxic.
§ Includes trace amounts of other hydroxymercapturic acids.
Amounts are expressed as percentages of the dose, ranges are given in parentheses and numbers of experiments are indicated by superscript numbers. ND indicates not detected; tr indicates trace amounts.

ethereal extracts of the acidified urine of dosed rabbits and rats revealed three sulphur-containing zones corresponding to cycloheptylmercapturic acid, 2-hydroxy and 3-hydroxycycloheptylmercapturic acids. These identifications were confirmed by GLC of the methylated metabolites which showed that both *cis*- and *trans*-(2-hydroxycycloheptyl)mercapturic acids were present and that the spot corresponding to 3-hydroxycycloheptylmercapturic acid contained substances giving two peaks corresponding to the two peaks of the synthetic material presumably corresponding to *cis*- and *trans*-isomers. In some experiments a further component with a longer retention time than that of the standard hydroxycycloheptylmercapturic acids synthesized was seen which may represent a 4-hydroxy isomer.

(*b*) *Cycloheptene.* With both rabbits and rats small amounts of cycloheptylmercapturic acid and the *cis*- and *trans*-2-hydroxy isomers were shown to be present. The major sulphur-containing metabolite consisted of a mixture of *cis*- and *trans*-(3-hydroxycycloheptyl)mercapturic acid.

(*c*) *Cycloheptene epoxide.* With both rabbit and rat a single sulphur-containing spot was detected by chromatography which corresponded to *cis*- or *trans*-(2-hydroxycycloheptyl)mercapturic acid. Analytical GLC confirmed that both isomers were present.

No sulphoxides were detected in the examination of the urines, or ethereal extracts of the urines, of rabbits or rats dosed with this series of compounds.

Experiments with rats fed with [^{35}S]*-labelled yeast*

Chromatograms of the urine of rats fed with [^{35}S]-labelled yeast and dosed with the above compounds were prepared. These were scanned and autoradiographs were prepared. In all cases the results confirmed those obtained by paper chromatography as described above and no further sulphur-containing metabolites were revealed.

Experiments with rat liver slices

The formation of cycloalkyl and hydroxycycloalkylmercapturic acids was detected when rat liver slices were incubated with the bromocycloalkanes studied but the hydroxylated mercapturic acids were not formed when the digests were saturated with carbon monoxide; however, cycloalkylmercapturic acids were formed under these conditions.

DISCUSSION

The results show that the bromocycloalkanes studied give rise in both rabbit and rat to mercapturic acids, the amounts formed decreasing with increasing ring size. The amount of cycloalkylmercapturic acid formed is very small, the sulphur-containing metabolites consisting chiefly of hydroxymercapturic acids of which the 3-hydroxy isomer is the major component. A similar pattern for the excretion of sulphur-containing metabolites after the administration of the cycloalkenes was observed, not more than trace amounts of cycloalkyl mercapturic acids being formed and the 3-hydroxymercapturic acid being the major metabolite. Again the percentage of the dose converted to mercapturic acid decreased from cyclopentene to cycloheptene. The corresponding cycloalkene epoxides were converted to 2-hydroxymercapturic acids, both *cis* and *trans* isomers being excreted in the case of cyclopentene and cycloheptene; after dosing with cyclohexene epoxide only traces of the *trans*-2-

hydroxycyclohexyl mercapturic acid were detected and it was found that the synthetic *trans*-2-hydroxy isomer was markedly unstable.

When administered to the rat all the compounds examined cause a fall in the level of the total glutathione in the liver, less marked with the bromocycloalkanes. This agrees with the generally accepted view that with those compounds which form mercapturic acids, the initial conjugation is with glutathione. The excretion of 2-hydroxymercapturic acids only, after dosing both rabbit and rat with the cycloalkene epoxides, suggests that a proportion of the unchanged epoxides reaches the liver and there combines with glutathione, the reaction presumably being catalysed by glutathione-S-epoxide transferase. Boyland and Williams[2] showed that cyclohexane epoxide was a substrate for this enzyme.

The pathway by which hydroxymercapturic acids are formed from bromocycloalkanes and cycloalkenes is less well defined. It was found that these compounds combine with cytochrome P_{450} to give difference spectra characteristic of Type I substrates. The bromocycloalkanes may therefore be hydroxylated by the microsomal hydroxylating system to give bromohydrins from which bromine is displaced by glutathione or the bromohydrin may conjugate with glucuronic acid or sulphate. It was previously reported[26] that the glucosiduronic acids formed are derivatives of 2-hydroxybromoalkanes and the present results suggest that the 3-hydroxy isomer is most readily converted to a hydroxymercapturic acid. Barnsley[4] has described the formation of 2-hydroxypropylmercapturic acid from 1-chloro-2-hydroxypropane and the bromohydrin derived from indene forms a hydroxymercapturic acid[28] so that mercapturic acid formation may be a general reaction of halogenohydrins. The possibility that a proportion of the administered bromocycloalkanes undergo dehydrobromination to cycloalkenes which then undergo hydroxylation and conjugation with glutathione cannot be excluded. Cycloalkenes produce a more marked fall in the total glutathione content of rat liver than do the bromocycloalkanes, that with cyclohexene being particularly marked and most rapid. Boyland and Chasseaud[27] found that cyclohex-2-en-1-one produced a similar marked and rapid fall in the level of glutathione in rat liver. Only a very small proportion of the dosed bromocycloalkanes or cycloalkenes is converted to cycloalkylmercapturic acid. It therefore seems likely that the direct conjugation of these compounds with glutathione occurs to a limited extent, and it is probable that hydroxylation occurs quickly. It is interesting that cyclohexane is an excellent substrate for hydroxylation by the P-450 complex and that cyclohexene forms the largest amount of hydroxymercapturic acid. The formation of cycloalkylmercapturic acids and their hydroxy derivatives was detected when bromocycloalkanes were incubated with rat liver slices and formation of hydroxymercapturic acids was prevented when the digests were saturated with carbon monoxide.

Grover and Sims[29] have shown that γ-benzene hexachloride in the rat undergoes dehydrochlorination to γ-pentachlorocyclohexene which then conjugates with glutathione, a chlorine atom being replaced by the glutathione radical. In flies and grass grubs Clark *et al.*[30] have shown that the removal of hydrogen chloride is unlikely to be the initial step in the metabolism of γ-benzene hexachloride and their evidence supports the view of Bradbury and Standen[31] that the initial metabolite is a pentachlorocyclohexyl glutathione.

The formation of sulphur-containing metabolites in no case accounts for more than

25 per cent of the administered dose of the compounds studied. In the rabbit the compounds all give rise to sulphate conjugates and with the exception of cyclopentene to glucosiduronic acids, the proportion increasing with increasing ring size. This is in contrast to the results[22] obtained with *n*-alkane derivatives, which form only small amounts of glucosiduronic acids not significantly increased with increasing chain length. The cycloalkenes gave rise to greater amounts of mercapturic acid than do the corresponding straight-chain 1-alkenes, the administration of which to rabbits results in the excretion of only traces of mercapturic acid.[32]

Acknowledgements—We are indebted to Dr. J. R. Majer of the Department of Chemistry, University of Birmingham for the mass spectrometry. We thank Miss S. Armstrong for technical assistance.

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